Asia-Pacific Journal of Science and Technology :Volume :30 Issue :05 Article ID :APST-30-05-09 Research Article

APST

Asia-Pacific Journal of Science and Technology

https//:www.tci-thaijo.org/index.php/APST/index

Published by Research and Innovation Department,
Khon Kaen University, Thailand

Efficiency of *Bacillus* spp. isolated from food waste compost to control leaf spot disease in green oak leaf lettuce

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Received 16 November 2024
Revised 26 February 2025
Accepted 22 April 2025

Abstract

Bacillus spp. are often used as bioactive agents to control plant diseases. *Corynespora cassiicola* is a fungus that causes a serious disease of salad vegetables known as leaf spot disease. In this study, 08 bacterial strains belonging to *Bacillus* spp, isolated from food waste compost, were tested for their ability to induce hemolysis on sheep blood agar and to inhibit *C. cassiicola* in a dual culture assay. The 8 strains included *B. subtillis* strain BS, *B. licheniformis* strain No.13, *B. amyloliquefqciens* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, *Lysinibacillus* sp. strain 3-BA, *B. altitudinis* strain No.20 and *B. licheniformis* LTWS strain No. 30. Two strains, *B. altitudinis* strain No. 20 and *B. licheniformis* LTWS strain No. 30 induced α-hemolysis, while the other 6 strains induced γ-hemolysis. Of the six non-hemolytic strains, *B. amyloliquefaciens* strain C2-1, *B. licheniformis* strain 2-BA and *Lysinibacillus* sp. strain 3-BA showed antifungal activity against *C. cassiicola* in the dual culture assay but *B. amyloliquefaciens* strain C2-1 inhibited *C. cassiicola* more strongly than the other two strains, with a percent inhibition of 69.38% after 9 days. *B. amyloliquefaciens* strain C2-1 was effective in suppressing the growth of *C. cassiicola* on the leaves of green oakleaf lettuce. The result of this study suggests that *B. amyloliquefaciens* strain C2-1 could be used as an alternative biocontrol agent against *C. cassiicola*.

Keywords: *Bacillus amyloliquefacien* strain C2-1, *Corynespora cassiicola*, dual culture assay, leaf spot disease, lettuce

1. Introduction

Lettuce (*Lactuca sativa* L.) is indeed valued as a nutrition leafy vegetable considered a healthy food. Its popularity as a salad or garnish [1,2] has led to its widespread cultivation for consumption and sale. In 2018, around 27 million tons of lettuce were produced worldwide [3]. Thailand exported 7.05 million kg of lettuce and chicory worth $4.94 million [4]. However, lettuce is susceptible to leaf spot disease caused by variety microorganisms, including the fungi *Cercospora sp*. The disease was first detected in 2004 on lettuces grown outdoors in nutrient film technique systems [5]. The spread of the disease is more common in the tropics and increases during the rainy season [6]. It causes small, circular to irregular brown spots with dark margins on lettuce leaves, leading to significant yield losses of up to 50%, depending on environmental conditions and control measures [7]. Chemical fungicides have been used to control leaf spots because they are a simple and convenient method of control. However, the use of chemicals leads to the formation of residues on the plants and pollutes the environment. Therefore, the search for more modern, environment-friendly approaches to plant disease control is interesting and leads to the use of biological products for safe biological control instead of chemicals.

Bacillus spp. has received great attention as bacterial biocontrol agents. They produce antagonistic agents with antifungal and antibacterial properties, siderophores, and endospores that can survive unfavorable environmental conditions [8, 9]. *Bacillus* spp. also have the ability to induce plant resistance to pathogens [10, 11]. *B. subtilis* LBF02 was shown to control lettuce leaf spot disease caused by *C. lactucae-sativae* with a growth inhibition of 80.82% [8]. *B. subtilis* B25, isolated from the rhizosphere of the banana plant, showed strong activity against *Fusarium oxysporum f.sp. cubense* and *C. cassiicola* [12]. *B. amyloliquefaciens* and *B. tequilensis* were effective antagonistic bacterial species in a dual culture technique that inhibited leaf spot disease due to *C. cassiicola* in cucumber [13]. Duy [14] isolated bacterial strains from the rhizosphere of the cucumber plant and tested their antifungal activity against *C. cassiicola* using a dual culture assay. The isolated bacterial strains exhibited fungal growth inhibition ranging from 39.8% to 62.6%.

In a previous study [15], five antagonistic *Bacillus* strains were isolated from food waste compost. *B. subtilis* strain BS, *B. amyloliquefaciens* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA and *Lysinibacillus* sp. strain 3-BA were tested for their efficacy against the rice pathogens *X. oryzae*, *F. fujikuroi* and *M. oryzae*. *B. amyloliquefaciens* strain C2-1 showed the highest inhibition percentage. Therefore, in this study, we further investigated the potential of these five strains alongside three additional strains, *B. licheniformis* strain No.13, *B. altitudinis* strain No.20 and *B. licheniformis* LTWS strain No. 30, isolated from food waste. The isolates were tested for hemolysis using the sheep blood agar method to assess toxicity to humans. The non-toxic isolates were then used in dual culture assays to assess their ability to inhibit the growth of *C. cassiicola*. The *Bacillus sp*. that showed the highest inhibitory activity was finally tested for its efficacy against *C. cassiicola* on green oak leaf lettuce. The selected *Bacillus* sp. could be used as an alternative strain for biological control of leaf spot disease caused by *Cercospora*.

2. Materials and methods

2.1 Biocontrol agents and pathogens

Eight bacterial strains belonging to *Bacillus* spp. were obtained from the Center for Genomes and Bioinformatics, Faculty of Science, Prince of Songkla University (PSU), Songkhla, Thailand, isolated from food waste compost. The strains were *B.subtilis* strain BS, *B. licheniformis* strain No.13, *B.altitudinis* strain No.20, *B. licheniformis* LTWS strain No.30, *B.amyloliquefaciens* strain C2-1, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus* sp. strain 3-BA. All bacterial strains were streaked on nutrient agar (NA) and incubated at 37 °C for 24 h to obtain single colonies for use as inocula. The pathogenic fungus, *C. cassiicola* PSU-PM-SK01, isolated from the leaf spots on lettuce (*Lactuca sativa*) [18], was obtained from Faculty of Natural Resources, PSU. A *C. cassiicola* PSU-PM-SK01 inoculum was cultured on potato dextrose agar (PDA) (Himedia, India) and incubated at 25 °C for 7 days.

2.2 Bacterial hemolysis

All eight *Bacillus* strains were cultured on sheep blood agar plates (5%) (Merck, Darmstadt, Germany) to determine hemolytic properties for the assessment of toxicity. Each strain was streaked on both the left and right sides of the plate and the plates were then incubated at 37 °C for 24 h until red blood cells began to decompose [13]. Hemolytic properties were classified as α-hemolysis, β-hemolysis and γ-hemolysis. The classifications were respectively defined by partial hydrolysis zones and green zones (α-hemolysis), clear zones around a colony (β-hemolysis), and no reaction (γ-hemolysis) [17].

2.3 Anti-fungal activity

The eight bacterial strains were cultivated in nutrient glucose broth (NGB) at 37 °C and 200 rpm until the cell suspension density (OD600) was 0.5. *C. cassiicola* was cultured on PDA (Himedia, India) and incubated at 25 °C for 7 days. Using a 0.5 cm diameter cork borer, a mycelial plug was taken from the edges of the growing fungal colony. The plug was placed in a fresh Petri dish about 2.5 cm in from the edge and a cell suspension of a *Bacillus* sp. was streaked as a thin line about 3 cm long down the opposite side of the Petri dish, 2.5 cm from the edge, before the plates were inoculated at 25 °C for 7 days [17]. The experiment was repeated three times and inhibition zones were measured on days 3, 5 and 7. The growth of the antagonists and the pathogen were measured as the radius of the colony to calculate the percentage inhibition of radial growth according to the formula in Equation 1 [19].

$$\textit{Percent inhibition of radial growth} = (R_1 - R_2) / R_1 \times 100 \quad (1)$$

where R_1 = radial growth (in mm) of the pathogen in the control (without biocontrol agent) and R_2 = radial growth (in mm) of the pathogen in the presence of the biocontrol agent

The *Bacillus* sp. that showed the highest percentage inhibition of radial growth was retested for its antagonistic activity and the zone of inhibition was measured on days 3, 5, 7 and 9. The anti-fungal efficacy of the strain was then tested against *C. cassiicola* on green oak leaf lettuce.

2.4 Anti-fungal activity on green oak leaf lettuce

Thirty-day-old lettuce plants (*Lactuca sativa*) grown in pots were purchased from Phu Than Plant Store, Songkhla, Thailand. The lettuce leaves were separated from the plants and washed with distilled water, drained, dried and blotted with sterile tissue paper. They were then placed in plastic boxes lined with damp tissue paper at the bottom to keep the humidity in the box high. To prevent the leaves from wilting, the leaf stems were covered with absorbent cotton moistened with water and wrapped in aluminum foil [20]. The selected *Bacillus* sp. was cultivated in NGB at 37 °C and 200 rpm until the cell suspension density reached 0.5 at OD600 and then used as the antagonistic bacterial strain. *C. cassiicola* was cultured in PDA for 7 days and then a 0.5 cm×0.5 cm piece of fungal mycelium was cut and used as the pathogen inoculum. A completely randomized design with four treatments and a total of five replicates for a total of 20 boxes was used for the study:

Treatment 1 (negative control): Each lettuce leaf was sprayed with 5 ml of distilled water

Treatment 2 (positive control): A piece of pathogen inoculum was placed on each lettuce leaf.

Treatment 3: A pathogen inoculum was placed on each lettuce leaf and then sprayed with 5 ml of bacterial inoculum

Treatment 4: Each lettuce leaf was sprayed with 5 ml of the bacterial inoculum.

The lettuce leaves were incubated in boxes at 25°C in an air-conditioned room. The disease incidence of was observed after 7 days of incubation and compared to the water-sprayed control. The percentage of infected leaf area was determined by visual assessment.

3. Results and discussion

3.1 Bacterial hemolysis

The hemolysis assay gives a rapid initial assessment of toxicity. Figure 1 showed that *B. amyloliquefaciens* strain C2-1, *B. subtillis* strain BS, *B. licheniformis* strain No.13, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA and *Lysinibacillus* sp. strain 3-BA showed no hemolytic activity against human blood in the blood agar medium and their hemolytic properties were classified as γ-hemolysis. These six strains were therefore considered safe in terms of hemolytic activity. Our results were in agreement with Saijai, [21] for *B. amyloliquefaciens*. Our results for *B. subtilis* strain BS were similar to that of Kim et al [22] for *B. subtilis* IDCC1101 and that of *B. licheniformis* strain No.13 was similar to that of Ebnetorab et al [23] for *B. licheniformis* Ahari. H2. Meanwhile, *B. altitudinis* strain No. 20 and *B. licheniformis* LTWS strain No. 30 produced green colonies that indicated partial decomposition of red blood cells. Their hemolytic properties were classified as α-hemolysis.

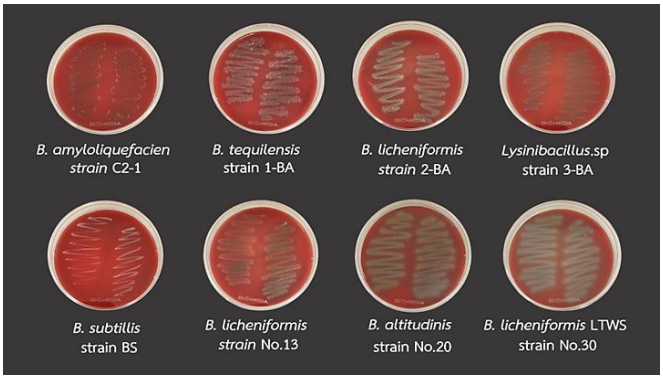

Figure 1 Hemolysis test of *B. amyloliquefaciens* strain C2-1, *B. subtillis* strain BS, *B. licheniformis* strain No.13, *B. tequilensis* strain 1-BA, *B. licheniformis* strain 2-BA, and *Lysinibacillus* sp strain 3-BA, *B. altitudinis* strain No.20 and *B. licheniformis* LTWS strain No.30.

3.2 Antifungal activity

Only three of the tested *Bacillus* spp, strains exhibited antifungal activity against the pathogenic fungus *C. cassiicola*. The three strains were *B. amyloliquefaciens* strain C2-1, *B. licheniformis* strain 2-BA and *Lysinibacillus* sp. strain 3-BA. They exhibited percent inhibitions of 55.14%, 25.00% and 28.57%, respectively, in dual culture assays at 7 days of incubation. *B. amyloliquefaciens* strain C2-1 inhibited the pathogen most and was therefore used in an additional antifungal assay over 9 days. On days 3, 5, 7 and 9, the growth area of the pathogen inoculated with *B. amyloliquefaciens* strain C2-1 was reduced compared to the control, while the *B. amyloliquefaciens* strain C2-1 surrounded the fungus on days 7 and 9 (Figure 2). The respective percent inhibitions were 16.67%, 39.47%, 55.00% and 69.38%. The inhibition of *C. cassiicola* by *B. amyloliquefaciens* strain C2-1 is consistent with Riddech et al [13], who reported that *B. amyloliquefaciens* can inhibit *C. cassiicola* in cucumber, and Bairwa et al [20] reported that *B. amyloliquefaciens* DSBA-11 reduces the growth of *C. cassiicola* in mung bean (Vigna radiata L. Wilczek). Monteiro et al [25] also reported that *B. amyloliquefaciens* exhibited significant antibacterial activity against *C. cassiicola* and showed 75.73% inhibition and 56% control of target spot disease in tomato plants in vitro. Therefore, *B. amyloliquefaciens* strain C2-1 is interesting as an alternative biocontrol agent against *C. cassiicola*. The morphological characteristics of *C. cassiicola* mycelia cultured with *B. amyloliquefaciens* strain C2-1 on the dual culture were observed after 9 days when the hyphae of *C. cassiicola* mycelia were examined under a simple light microscope (at 100X magnification) (Figure 3). The mycelia appeared shrunken and distorted, whereas the mycelia in the control were rather elongated. The abnormal mycelia at the border were consistent with the morphological characteristics of mycelia of rice fungal pathogens in dual culture plates with *B. amyloliquefaciens* strain C2-1 [15]. The abnormal morphology of the mycelia could be due to the tight adhesion of *B. amyloliquefaciens* strain C2-1 to the mycelia, resulting in stunted fibers and thus wrinkling on the surface [26]. *B. amyloliquefaciens* strain C2-1 was therefore used for further studies of the inhibition of *C. cassiicola* on lettuce leaves.

Figure 2 *B. amyloliquefaciens* strain C2-1 inhibited the growth of *C.cassiicola* in a dual culture assay.

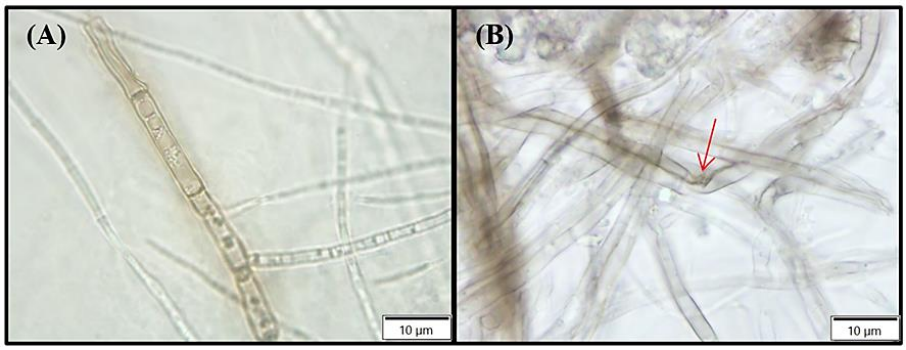

Figure 3 Morphology of *C. cassiicola* mycelia on dual culture plates after 9 days of incubation: (A) control *C. cassiicola*; (B) *C. cassiicola* + *B. amyloliquefaciens* strain C2-1. The scale corresponds to 10 µm on the image. A compound microscope was used at 100x magnification. The arrow points to a swollen balloon-like abnormality.

3.3 Antifungal activity on green oak leaf lettuce

The inhibition of *C. cassiicola* on green oak lettuce leaves by *B. amyloliquefaciens* strain C2-1 is shown in Figure 4. The appearance of the leaves sprayed with distilled water or with *B. amyloliquefaciens* strain C2-1 did not change, but leaves exposed to agar pieces of *C. cassiicola* showed growths of fungal hyphae around the agar pieces. No hyphal growth was observed around the pathogenic fungus on leaves that were sprayed with *B. amyloliquefaciens* C2-1. This result showed that *B. amyloliquefaciens* strain C2-1 was able to inhibit the spread of *C. cassiicola* on green oak leaf lettuce.

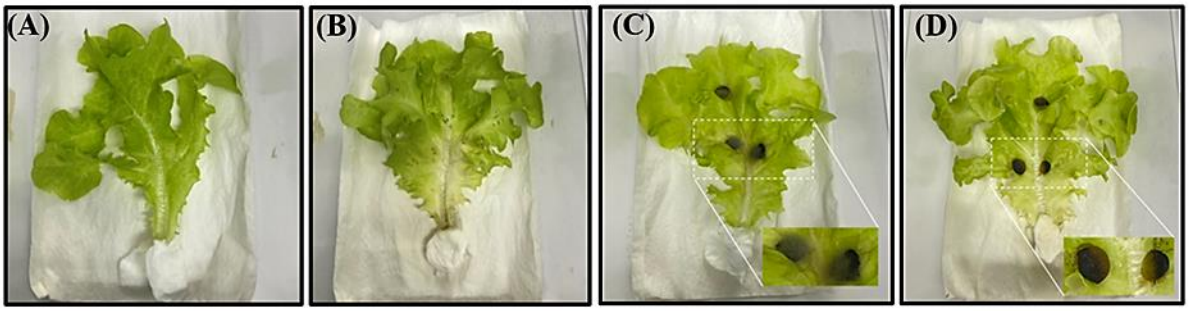

Figure 4 Physical appearance of *B. amyloliquefaciens* strain C2-1 against *C. cassiicola* on green oak leaf lettuce: Green oak leaf lettuce sprayed with (A) distilled water; (B) with B. *amyloliquefaciens* strain C2-1: Green oak leaf lettuce exposed to (C) *C. cassiicola*; (D) exposed to *C. cassiicola* and sprayed with *B. amyloliquefaciens* strain C2-1.

4. Conclusions

Eight strains of *Bacillus* spp. isolated from food waste compost were tested for hemolytic properties and inhibitory activity against a pathogenic strain of *C. cassiicola*, which causes leaf spot disease in salad vegetables. *B. amyloliquefaciens* strain C2-1, *B. subtillis* strain BS, *B. licheniformis* strain No.13, *B. tequilensis* 1-BA, *B. licheniformis* strain 2-BA and *Lysinibacillus* sp. strain 3-BA did not induce hemolysis while *B. altitudinis* strain No. 20 and *B. licheniformis* LTWS strain No. 30 induced alpha hemolysis. *B. amyloliquefaciens* strain C2-1 showed the strongest inhibitory activity against *C. cassiicola*, with a percentage inhibition of 69.38% after 9 days. On green oak leaf lettuce leaves exposed to *C. cassiicola* and subsequently sprayed with *B. amyloliquefacien* strain C2-1, no growth of fungal hyphae was observed 7 days after treatment. Thus, *B. amyloliquefaciens* strain C2-1 shows potential for use as a biocontrol against leaf spot disease in lettuce and is safe for humans. Further investigations could determine the optimal dose and shelf life of *B. amyloliquefaciens* strain C2-1 in home trials at different locations.

5. Conflicts of Interest

The authors declare no conflict of interest.

6. References

[1] To-Anun C, Hidayat I. and Meeboon J. *Genus Cercospora* in Thailand: Taxonomy and Phylogeny (with a Dichotomous Key to Species). Plant Pathol Quarant. 2011; 1: 11–87.

[2] Dupont S, Mondi Z, Willamson G and Price K. Effect of variety, processing, and storage on the flavonoid glycoside and composition of lettuce and chicory. J Agric Food Chem. 2000; 48: 3957–3964.

[3] Sronsri C, Sittipol W, and U-yen K. Quantity and quality of lettuce (*Lactuca sativa* L.) grown by a circulating hydroponic method with a Halbach array magnetizer. J Agric Food Chem. 2022: 108: 104460.

[4] DiverCity Times. Thailand export volume of lettuce and chicory: Key data and insights for global markets. 2025

[5] Koohakan P, Jeanaksorn T, and Nuntagij I. Major diseases of lettuce grown by commercial nutrient film technique in Thailand. KMITL Sci Technol J. 2008; 8:56-63.

[6] Agrios GN, Plant pathology (5th ed.) Elsevier Academic Press. London.2004; 952.

[7] Mirah AA I. *Cercospora Canescens*: Cause of leaf spot disease on lettuce crop in Bali province, Indonesia. SABRAO J Breed Genet. 2023; *55*(6):2006–2016.

[8] Srimai K, and Angsana A. *Bacillus subtilis* LBF02 as biocontrol agent against leaf spot diseases caused by *Cercospora Lactucae-Sativae* in lettuce. J Agric Sci. 2014;6(3):151-158.

[9] Khan A R, Mustafa A, Hyder S, Valipour M, Rizvi ZF, Gondal A S, Yousuf Z, Iqbal R, and Daraz U. *Bacillus* spp. as bioagents: uses and application for sustainable agriculture. Biology. 2022;11(12): 1763.

[10] Hemsanit N and Pratuangwong S. *Pseudomonas fluorescens* SP007s stimulate kale to produce salicylic acid to resist golden leaf margin disease, The 47th Kasetsart University Annual Conference. Plant Sci Bangkok. 2009; 611-620.

[11] Kloepper W. Ryu JCM and Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology.2004; 94: 1259-1266.

[12] Tan Z, Lin B, Zhang R. A novel antifungal protein of *Bacillus subtilis* B25. SpringerPlus. 2013; 2(1):543.

[13] Riddech N, Sritongon N, Phibunwatthanowong, T. Production of plant growth promoting antagonistic rhizobacteria to promote cucumber growth and control leaf spot disease (*Corynespora Cassiicola*). Chiang Mai J Sci. 2016*;*44(1): 72-82.

[14] Duy LQ. Evaluation of antifungal activity against *Corynespora cassiicola* by bacteria isolated from soil in the root zone of cucumber plants. Biodiversitas. 2023; 44(12): 6584-6591.

[15] Meehae U, Kumngen A, Chotigeat W, Suwannarat S, Anuchan S, Whankaew S, Leunram S, Karrila S, and Iewkittayakorn J. Potential of *Bacillus* spp. isolated from food waste compost for controlling rice diseases. Sains Malaysiana. 2023*;* 52(7): 1999–2007.

[16] Silaram M. Method of selecting spherical gram-Positive bacteria, Faculty of medicine and public health. Ubon Ratchathani Univ J. 2018; 3: 23-25.

[17] Shi Q, Xinrui W, Zijing J, Biqin L, Changwei L, Hongning W and Hong L. Technological and safety characterization of *Kocuria Rhizophila* isolates from traditional ethnic dry-cured ham of Nuodeng, Southwest China. Front Microbiol. 2021; 44(12): 761019.

[18] Chairin T, Pornsuriya C, Thaochan N, and Sunpapao A. *Corynespora cassiicola* causes leaf spot disease on lettuce (*Lactuca sativa*) cultivated in hydroponic systems in Thailand. Australas Plant Dis Notes. 2017; 12(1): s13314-13317.

[19] Boonrayong M, Somsirikul P, Klodpeng N, and Orawan P. Bioactive potential of *Myrothecium inundatum* against microbial plant pathogens and free-floating aquatic weeds. Ramkhamhaeng Int J Sci Technol. 2023; 6(1): 22-29.

[20] Chomduang J, Wichitrakulthaworn K and Khomma W. Application of antagonistic microorganisms for the prevention and control of frog-eye leaf spot disease of lettuce. Royal Project Foundation. 2015.

[21] Saijai K. Antibacterial activity of *Bacillus* spp. against foodborne pathogens. YRU J Sci Technol. 2016; 17(2):51-59

[22] Kim SH, Yehuala, GA, Bang WY, Yang J, H.Jung Y. and Park MK, Safety evaluation of *Bacillus subtilis* IDCC1101, newly isolated from Cheonggukjang, for industrial applications. Microorganisms. 2022; 10(12): 2494.

[23] Ebnetorab SMA, Ahari H, and Kakoolaki S, Isolation, biochemical and molecular detection of *Bacillus subtilis* and *Bacillus licheniformis* from the digestive system of rainbow trout (*Oncorhynchus mykiss*) and its inhibitory effect on *Aeromonas hydrophila*, Iran J Fish Sci. 2020; 19(6): 2824-2845,

[24] Bairwa NK, Jambhulkar PP, Sushmitha V, Arya M, Manjunatha N, Bajpai R, Singh D, Mani C, S. Kumar,Chaturvedi, K. Sushil., and D. Lakshman. Evaluation of fungicides and bacterial antagonists for management of *Corynespora* leaf spot on mungbean (*Vigna radiata* L. *Wilczek*), Arch Phytopathol Pflanzenschutz. 2022; 55(4): 433–453,

[25] Monteiro GP, Silveira TM, Blind AD, Figueirêdo P, Nevesd AF, Santos R. *Bacillus amyloliquefaciens*, *Microbacterium resistens* and *Stenotrophomonas maltophilia* Biocontrol of *Corynespora cassiicola* in Tomato Culture. Seven Editora. 2023;9(1):4677–4697.

[26] Sungtong S, Pengnoo A and Boonyapipat P. Efficacy of *Bacillus* spp. in controlling soilborne pathogen *Rigidoporus microporus* under control conditions. Songklanakarin J Plant Sci (SJPS). 2021; 8(1): 34-432021 (in Thai).